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Suspended culture of the sea cucumber *Apostichopus japonicus* below a Pacific oyster raft – potential for integrated multi-trophic aquaculture

(Running title: Co-culture of Pacific oysters and sea cucumbers)

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Abstract

In order to discuss the possibility of co-culturing Pacific oyster with the sea cucumber *Apostichopus japonicus*, a field experiment was conducted in an oyster farm. *A. japonicus* juveniles (mean wet weight, 0.08 g) were cultured below an oyster raft and at a control station for 216 days, and the wet weight and stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$) were analyzed together with settling organic matter (OM) collected by sediment traps. All sea cucumbers cultured below the raft survived (survival rate, 100%), while at the control station 1 individual disappeared (96%). During 216 days, the juveniles at the oyster and control stations grew to a mean weight of 5.5 g and 2.6 g, attaining respective specific growth rates of 2.0% and 1.6% (paired *t*-test, $P < 0.001$). Settlement rates of carbon and nitrogen at the oyster station were ~5 times larger than those at the control station. The stable isotope analysis showed that settling OM at both stations originated from coastal phytoplankton and that phytoplankton represented the primary food source for *A. japonicus*. The rapid growth of *A. japonicus* at the oyster station was concluded to be due to the abundant supply of oyster biodeposits, which could be ingested by this species.

Key words: sea cucumber, *Apostichopus japonicus*, Pacific oyster, co-culture, stable isotope

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Introduction

In shellfish farms, faeces and pseudofaeces from the cultured bivalves and fouling organisms such as mussels and ascidians, which originate in autochthonous particulate organic matter (OM) in seawater, settle onto the seabed. Such biodeposits result in elevated levels in OM, phaeopigments and sulphides in the sediment and in deoxygenation of the bottom water (e.g., Kusuki 1981). Such localized accumulation of OM often produces negative consequences for farm management by incidental mass mortality of cultured bivalves (reviewed by Mori 1999). Preventing localized accumulation of biodeposits is important to achieve environmentally responsible aquaculture.

Integrated multi-trophic aquaculture (IMTA) has the potential to reduce waste loading and environmental impacts and to increase the efficiency and productivity of monoculture systems (reviewed by Neori, Chopin, Troell, Buschmann, Kraemer, Halling, Shpigel & Yarish 2004). Sea cucumbers are detritus feeders which ingest sediment with OM including bacteria, protozoa, diatoms and plant and animal detritus (e.g., Yingst 1976). As a result of their feeding behavior, they would be a good candidate for co-culture in IMTA systems with either cultured finfish (Ahlgren 1998; Yokoyama 2013), bivalves (Zhou, Yang, Liu, Yuan, Mao, Liu, Xu & Zhang 2006; Slater & Carton 2007; Paltzat, Pearce, Barnes & McKinley 2008), abalone (Kang, Kwon & Kim 2003), shrimp (Pitt, Duy, Duy & Long 2004) or jellyfish (Ren, Dong, Wang, Gao & Jiang 2012).

The Japanese common sea cucumber *Apostichopus japonicus* (Selenka) is a valuable species in many parts of Asia due to its high meat quality for traditional food and purported medicinal properties (Huiling, Mengqing, Jingping & Bijuan 2004), which has led to overfishing and subsequently the need for the development of methods for aquaculture and stock enhancement in Asia (Ito 1995; Chen 2004). Zhou *et al*. (2006) reported that cultured *A. japonicus* in hanging scallop lantern nets fed on biodeposits, grew and survived well resulting

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in a reduction in bivalve waste. Yokoyama (2013) also reported that *A. japonicus* juveniles grew to marketable sizes by assimilating fish farm waste, while cultured below fish pens. IMTA operations in which *A. japonicus* grow along with co-cultured Pacific oysters could also possibly have the same effects as those found in Zhou *et al*. (2006) and Yokoyama (2013). This study was designed to compare the survivorship and growth of *A. japonicus* between juveniles cultured below a raft of cultured Pacific oysters (*Crassostrea gigas*) and those at a control site over a 7-month period and to examine the potential use of biodeposits from oyster farming as a diet of the sea cucumbers using the stable isotope analysis.

Materials and methods

Sea cucumbers used in the experiment

It has been recognized that there are three body color types of *A. japonicus*, i.e., green, black and red types (e.g., Choe & Ohshima 1961). In the present study, juveniles of the green type of *A. japonicus*, which were bred artificially at the Hakuyo Nursery Farm (Shima, Japan), were used for the experiment. Hakuyo Nursery Farm fertilized eggs on May 2, 2011 and confirmed settled juveniles on May 13. The juveniles were transferred to a tank in the laboratory on July 20, and maintained by feeding commercial food (LIVIC-BW, Eiken-Shoji Co. Ltd., Kandajinbou, Tokyo, Japan) until the start of the field experiment.

Culture experiment

A culture experiment was conducted in an aquaculture ground of Pacific oyster in Gokasho Bay, which is located on the Pacific coast of middle Japan. Prior to the culture experiment, the juveniles were starved for 24 h and weighed individually after external water was removed from specimens by drying them on a paper towel. Among them, five sea cucumbers were sampled for analysis of the initial $\delta^{13}C$ and $\delta^{15}N$. The culture experiment started on August 9, 2011. Twenty-four juveniles were used for the culture experiment at an oyster station

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(34°20'29"N, 136°41'4"E), where approximately 10 000 Pacific oysters were cultured along 100 lines (length, 4.5 m) under a raft (5.5 × 6.5 m), and at a control station (34°20'25"N, 136°40'57"E), using a similar type of raft under which no oysters were cultured. The juveniles were each placed individually in a culture vessel, which was made of polyvinyl chloride (PVC) gray tube (Fig. 1). Both ends of the tube were covered with nylon net sheets using plastic tape. A piece of nylon net sheet (mesh size, 1 mm) was put inside each culture vessel to act as an additional substrate to enable a greater surface area over which particulate OM can settle and subsequently enable more potential food for the sea cucumbers. As the sea cucumbers grew, the sizes of the vessel, nylon net sheets and mesh of the net sheets were changed in the manner shown in Yokoyama (2013). The culture vessels were placed in the containers (45 × 31 × 17 cm) and were maintained at a depth of 6.5 m from the sea surface below the oyster raft. During the experiment, wet weights of the sea cucumbers were measured once a month, and water temperature in the culture containers was continuously monitored by logger-style thermometers (MDS-MK V/T, Alec Electronics, Kobe, Japan). Culture in the PVC tubes was finished on March 12, 2012 after 216 days, then all individuals were weighed after 24 h starvation, and five sea cucumbers were used for the $\delta^{13}C$ and $\delta^{15}N$ analysis.

Fig. 1

Settling OM was collected once a month from the oyster and control stations by deploying sediment traps. Each sediment trap was equipped with four acrylic cylinders (74 mm inner diameter and 300 mm high).The mouths of the traps were set at the same depth (6.5 m) as the culture containers.

Sample collection and determination of isotopic compositions

Epidermis and body muscle of sea cucumbers were dissected and soaked in 1.2 N HCl for a few minutes to remove traces of carbonates, and when CO_2 bubbles were no longer observed, rinsed with distilled water, dried at 60°C, ground to a powder, and analyzed for the stable

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carbon and nitrogen isotope ratios ($\delta^{13}C$, $\delta^{15}N$). The sediment trap material was sieved on a 1 mm mesh to remove benthic animals, filtered through a Nuclepore polycarbonate track-etch membrane filter (pore size, 0.8 µm), soaked in 1.2N HCl for 1 h, rinsed with distilled water, dried at 60°C, weighed, powdered and used for total organic carbon (TOC), total nitrogen (TN) and isotopic analyses. A mass spectrometer (MAT 252, Finnigan MAT) coupled online via a Finnigan ConFlo II interface with the elemental analyzer was used for the elemental and isotopic analyses.

Data calculation and statistical analysis

The specific growth rate (SGR, %) of *A. japonicus* was calculated as: SGR = 100 × [ln (W_t/W_0)]/t, where W_t is the wet weight after t days culture and W_0 is the initial weight. The contribution ratio (CR%) of each food source to the diet to *A. japonicus* was given based on isotopic compositions of this species and its possible food sources using the model presented in Yokoyama (2013).

To test statistical differences in the settlement rates of settling OM and wet weight and SGR of *A. japonicus* between the two stations, a two sample *t*-test was used.

Results

Water temperature and settlement rates of organic matter

Water temperature at the oyster station ranged from 10.9°C on February 20, 2012 to 27.1°C on September 21, 2010 (Fig. 2). There was no difference in water temperature between the 2 stations.

Fig. 2

Settlement rates of TOC and TN at the oyster station during the culture period (range of a monthly mean = 0.51–1.96 g C m^{-2} d^{-1} and 0.07–0.26 g N m^{-2} d^{-1}; total mean ± SD = 1.37 ± 0.55 g C m^{-2} d^{-1} and 0.18 ± 0.07 g N m^{-2} d^{-1}, n = 32) were much higher than those at the control station (0.04–0.67 g C m^{-2} d^{-1} and 0.01–0.09 g N m^{-2} d^{-1}; 0.29 ± 0.19 g C m^{-2} d^{-1} and

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0.04 ± 0.03 g N m^{-2} d^{-1}, n = 30) (Fig. 3a and b). The differences in the settlement rates of TOC and TN between the oyster farm and control stations were significant (paired t-test, P < 0.001) on all sampling occasions. The monthly $\delta^{13}C$ mean of settling OM ranged from –22.1‰ to –20.5‰ (total mean ± SD = –21.6 ± 0.5‰, n = 32) for the oyster station and from –21.9‰ to –20.4‰ (total mean ± SD = –21.5 ± 0.5‰, n = 32) for the control station (Fig. 3c). The monthly $\delta^{15}N$ mean of settling OM ranged from 4.8‰ to 7.8‰ (total mean ± SD = 5.9 ± 0.9‰, n = 32) for the oyster station and from 3.5‰ to 8.1‰ (6.0 ± 1.4‰, n = 32) for the control station (Fig. 3d). Significant differences (paired t-test, P < 0.05) were found between the 2 stations in the $\delta^{13}C$ on 2 occasions and in the $\delta^{15}N$ on 5 out of the 8 sampling occasions, however there was no consistent relationship between the two stations throughout the sampling period.

Fig. 3

Survival and growth of sea cucumbers

With the exception of one individual that was lost at the control station (survival rate, 96%) within the first month (28 days), all other sea cucumbers survived the duration of the trial at both locations.

The range and mean ± SD values of the initial wet weight of the sea cucumbers were 0.07–0.10 g and 0.08 ± 0.01 g (n = 24) for the oyster and control stations. Similarly final range and mean values were 4.0–7.8 g and 5.5 ± 1.2 g (n = 24) for the oyster station, and 1.2–3.5 g and 2.6 ± 0.7 g (n = 23) for the control station, respectively (Fig. 4a and b). During the culture period of 216 days, the sea cucumbers at the oyster station increased 45- to 108-fold in weight, while those at the control station showed a 15- to 50-fold increase. The range and mean ± SD values of SGR during this period were 1.8–2.2% and 2.0 ± 0.1% for the oyster station (n = 24), and 1.3–1.8% and 1.6 ± 0.2% for the control station (n = 23). There was a significant difference in SGR between the two stations (paired t-test, P < 0.001). At the outset of the trial, SGR at both stations was high (>5%). Thereafter, SGR at the control site

Fig. 4

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decreased, while the oyster station remained high, resulting in the large difference in the final weight between the two stations.

Isotopic composition of sea cucumbers

The $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SD) of sea cucumbers just before the field experiment were –14.1 ± 0.3‰ (n = 5) and 5.6 ± 0.5‰ (n = 5), respectively (Fig. 5). The $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SD) of the sea cucumbers after the 216-day culture below the oyster raft were –16.6 ± 0.2‰ (n = 5) and 8.6 ± 0.2‰ (n = 5), while those of the sea cucumbers cultured at the control station were –15.9 ± 0.1‰ (n = 5) and 8.8 ± 0.2‰ (n = 5), respectively. The sea cucumbers cultured at the oyster station were significantly reduced in ^{13}C (paired t-test, P < 0.001) by an average of 0.7‰ relative to those at the control station, whereas there was no significant difference (P = 0.26) in the $\delta^{15}N$ between the two stations.

Fig. 5

Discussion

When adopting the stable isotope technique to estimate food sources for the sea cucumbers, it is necessary to define isotopic compositions of the possible food sources, which include settling OM and primary producers such as terrestrial C_3 plants, coastal phytoplankton, seaweeds and epipelic and epilithic microalgae. Assuming that the $\delta^{13}C$ and $\delta^{15}N$ values of the sea cucumbers sampled in March reflect only the isotopic compositions of the diets for the 3 months prior to the collection (from December to March) due to their rapid growth, mean isotopic values for food sources during this period were evaluated. The primary producers in Gokasho Bay have been analyzed in previous studies; therefore, literature $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SD) collected during a period from December to March in different years, –26.5 ± 0.8‰ (n = 4) and 0.8 ± 1.4‰ (n = 4) for terrestrial C_3 plants, which were collected as riverine OM, –21.1 ± 0.7‰ (n = 4) and 5.1 ± 0.9‰ (n = 4) for coastal phytoplankton, which was collected as particulate OM in seawater, –15.0 ± 2.9‰ (n = 129) and 8.1 ± 1.0‰ (n =

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129) for seaweeds, –13.1 ± 3.0‰ (n = 4) and 5.3 ± 0.8‰ (n = 4) for epipelic microalgae (Yokoyama & Ishihi 2003), –19.8‰ ± 0.9‰ (n = 8) and 6.8 ± 1.8‰ (n = 8) for epilithic microalgae (Yokoyama & Ishihi 2006), were used (Fig. 5).

Among possible food sources for the sea cucumbers, settling OM was analyzed for their isotopic compositions. Mean (±SD) $\delta^{13}C$ and $\delta^{15}N$ values of settling OM collected from December to March were –21.4 ± 0.5‰ (n = 16) and 5.5 ± 0.6‰ (n = 16) at the oyster station, and –21.4 ± 0.6‰ (n = 16) and 5.9 ± 0.8‰ (n = 16) at the control station, respectively. These values were close to the values of coastal phytoplankton, indicating that the majority of settling OM at the two stations originated from coastal phytoplankton. The observed high levels of the settlement ranges of OM at the oyster station, therefore, are concluded to be composed mainly of biodeposits from the oyster culture, which mainly originated, through the filter feeding of the oysters, from coastal phytoplankton.

The $\delta^{13}C$ and $\delta^{15}N$ values of the estimated diet for cultured *A. japonicus*, which can be calculated based on the specific isotopic fractionation (2.6‰ for ^{13}C and 3.8‰ for ^{15}N, Yokoyama, 2013), are –19.2‰ and 4.8‰ for the oyster station and –18.5‰ and 5.0‰ for the control station. These values fell between those of coastal phytoplankton and epipelic microalgae (Fig. 5). These findings suggest that the diet of the sea cucumbers was a mixture of the OM from these two sources and that other potential sources such as terrestrial C_3 plants, epilithic microalgae and seaweeds were not utilized as an important food source. The calculation using the two-source model (Yokoyama 2013) indicated that the contribution ratio of coastal phytoplankton to the growth of *A. japonicus* was 76% for individuals cultured below the oyster raft and 68% for those at the control station.

The estimation of diet depends largely on the isotopic fractionation and variability in isotopic values of potential food sources. In recent years, it is increasingly apparent that fractionation is species-specific and that the accepted fractionation values, 0 to 1‰

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enrichment for ^{13}C and 3 to 4‰ enrichment for ^{15}N per trophic level, may not be universally applicable (e.g., McCutchan, Lewis, Kendall & McGrath 2003). The present study adopted the 2.6‰ enrichment for ^{13}C and 3.8‰ enrichment for ^{15}N as the fractionation of the diet-tissue fractionation of *A. japonicus* based on the laboratory experiment by Yokoyama (2013). Slater & Carton (2010) assumed the ^{13}C fractionation of 4‰ for *Australostichopus mollis* inhabiting a mussel farm, which was estimated to incorporate mussel biodeposits. If this ^{13}C fractionation value is applicable to *A. japonicus*, coastal phytoplankton would be the exclusive diet for *A. japonicus* at the oyster station, accounting for the contribution ratio of 94%. On the other hand, the isotopic compositions of primary producers often show large temporal and spatial variability (e.g., Gearing, Gearing, Rudnick, Requejo & Hutchins 1984). In Gokasho Bay, epilithic microalgae had enriched $\delta^{13}C$ value of –14.6‰ and reduced $\delta^{15}N$ value of 3.6‰ temporally (Yokoyama & Ishihi 2006). Supposing that epilithic microalgae exhibit such isotopic compositions for a certain period of time, epilithic microalgae, which are supposed to grow on the surface of the culture vessels and mesh nets, may play a certain role for the nutrition of *A. japonicus* (contribution ratio, 29%). Even if epipelic and/or epilithic microalgae contribute to its diet, coastal phytoplankton could represent the primary food source for the sea cucumbers cultured below the oyster raft and they could grew rapidly by ingesting the oyster biodeposits.

Suspended cage culture of sea cucumbers in coastal waters has been attempted several times (Ito 1995; Paltzat *et al*. 2008; Zhou *et al*. 2006; Yokoyama 2013; Yu, Hu, Zhou, Li & Peng 2013). This method achieved extremely high survival rates, that is, 96% of juvenile *A. japonicus* during the culture period of 238 days and 100% (307 days) of subadult *A. japonicus* (Yokoyama 2013) and 100% (80 days) of *Holothuria leucospilota* (Yu *et al*. 2013). It is probable that the high survival rate resulted from the capability of avoiding low salinity, high temperature and deoxygenated conditions as well as benthic predators. The cage culture,

Table 1

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however, has limitations related to living space and diets. Successful cage culture needs enough nutrition in the settling faecal material to support the growth of the sea cucumbers.

Yokoyama (2013) conducted a culture experiment of *A. japonicus* juveniles below a fish pen using the same method adopted in the present study and found that the juveniles having a mean wet weight of 0.12 g on Oct 13, 2010 grew up to a mean of 7.4 g during 168 days, accounting for a SGR of 2.6 ± 0.4% (mean ± SD, n = 46), which is significantly (paired *t*-test, $P < 0.001$) higher than the rate found at the oyster station in this study (2.0 ± 0.1%, n = 24) (Table 1). The settlement rate of carbon below the fish pen (0.67 ± 0.34 g C m^{-2} d^{-1}, n = 44) was smaller than the rate at the oyster station (1.40 ± 0.50 g C m^{-2} d^{-1}, n = 32), however, the sea cucumbers could grow rapidly by incorporating the dietary materials that had a high nutritional value. The stable isotope analysis showed that the juveniles below the fish pen assimilated C_3 plant material in fish feed such as wheat flour and defatted soybean meal, suggesting that these items have a high nutritional value. The growth rate at the oyster station was lower than the rate at the fish farm station; however, all sea cucumbers survived and grew faster than those at the control station (Table 1).

It has been pointed out that biodeposits of filter-feeding bivalves are potentially an available food source for sea cucumbers. Paltzat *et al.* (2008) found the successful utilization of biodeposits from cultured Pacific oysters by the sea cucumber *Parastichopus californicus*, which was cultured in trays deployed beneath oyster rafts. Tank-based feeding experiments of *Australostichopus mollis* also showed that the sea cucumbers consumed mussel farm-impacted sediments and reduced the accumulation of organic carbon and phytopigments (Slater & Carton 2007, 2009). Slater, Jeffs & Carton (2009) showed that fresh mussel faeces was a more suitable diet for this species than seaweed and natural sediment diets. Yuan, Yang, Zhou, Mao, Zhang & Liu (2006) suggested that fresh feces is more nutritious than dried faeces due to the presence of suitable bacteria. The suspended cage culture below oyster rafts,

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therefore, can be considered to provide a favourable habitat for sea cucumbers from the viewpoint of the quality and quantity of diets as well as the capability of avoiding unfavourable environments and benthic predators.

In conclusion, *A. japonicus* juveniles cultured below the Pacific oyster raft grew well, and exhibited the high survival rate (100%), indicating the possibility of co-culture of oysters and sea cucumbers, which will serve for bioremediation and also enable additional income from growing sea cucumber juveniles to larger sizes. Large-scale trials should be conducted to establish the IMTA system which is composed of Pacific oysters and *A. japonicus*.

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Table 1. Comparison of the growth *Apostichopus japonicus* cultured below the fish pen and at the 2 stations in the oyster farm.

	Fish pen*
Culture period	Oct 13, 2010 to March 30, 2011 (168 days)
Aug 9, 2011 to March 12, 2012 (216 days)	
Initial wet wt (g, mean ± SD)	0.12 ± 0.10 (n = 48)
Final wet wt (g, mean ± SD)	7.4 ± 2.3 (n = 46)
Specific growth rate (%, mean ± SD)	2.6 ± 0.4 (n = 46)
Settlement rate of Carbon (g C m^{-2} d^{-1}, mean ± SD)	0.67 ± 0.34 (n = 44)

* Data cited from Yokoyama (2013).
** Data collected from the 2 station below the oyster raft (the oyster station) and vacant raft (the control station) in this study.

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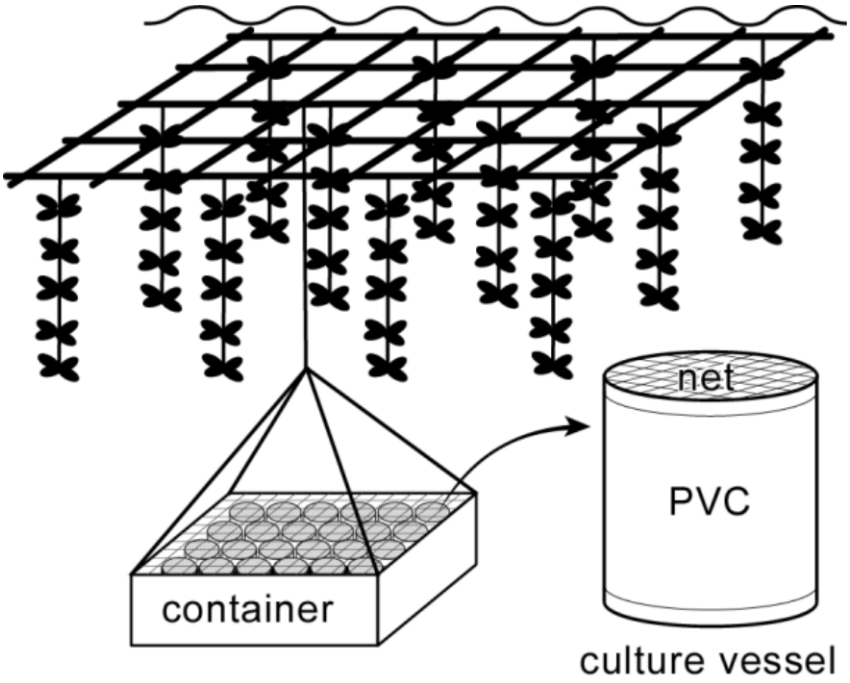

Fig. 1. Arrangement of the device for culturing *Apostichopus japonicus* below a Pacific oyster raft.

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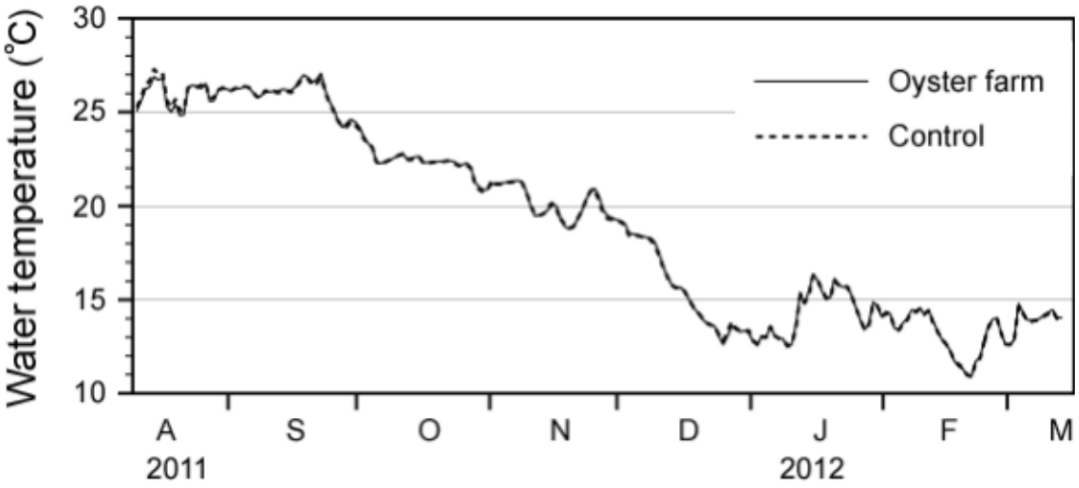

Fig. 2. Temporal fluctuations in water temperature at the oyster and control stations.

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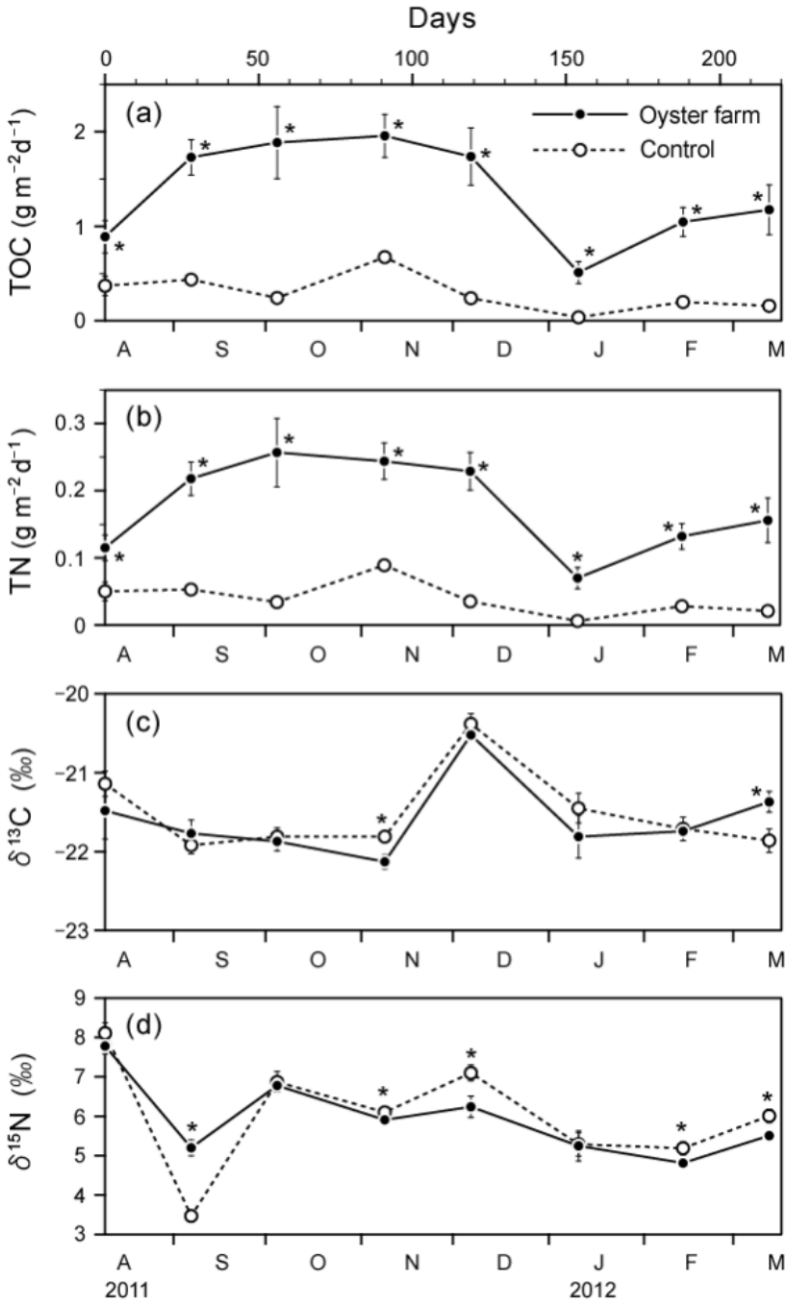

Fig. 3.Settlement rates of total organic carbon (a), total nitrogen (b), and stable carbon (c) and nitrogen (d) isotope ratios of settling organic matter. Asterisks show values with a significant difference ($P < 0.05$) between the oyster and control stations. Error bars are SD.

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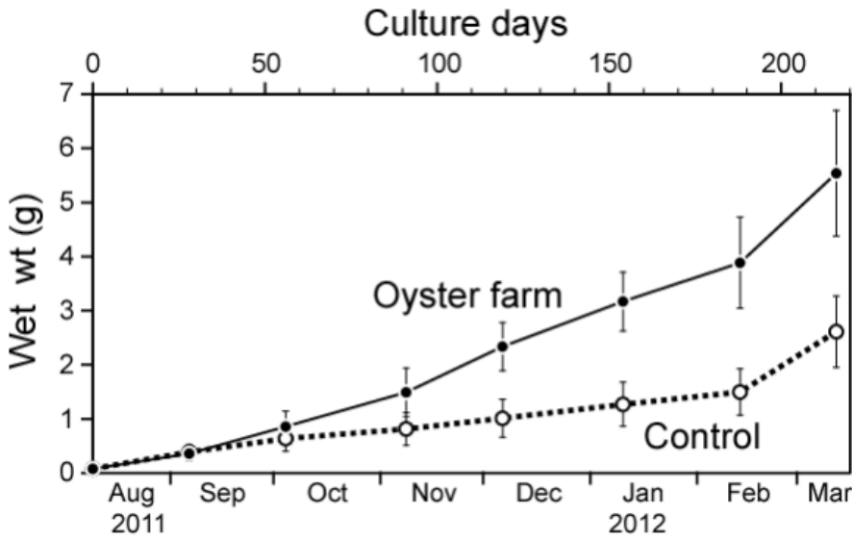

Fig. 4. Growth of *Apostichopus japonicus* during 216 days culture period at the oyster and control stations, in terms of monthly mean wet weight. Error bars are SD.

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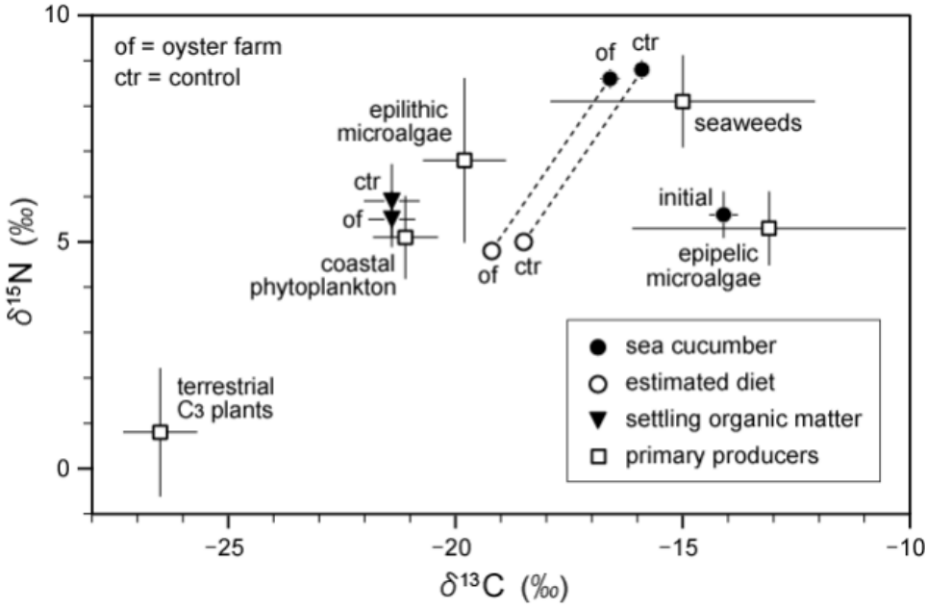

Fig. 5. Dual isotope plot of $\delta^{13}C$ and $\delta^{15}N$ (mean ± SD) for *Apostichopus japonicus*, its estimated diet and potential food sources, showing isotopic values of all components. Broken lines show the animal-diet relationship.